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Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation U.K. 7687697001

4. Title of the invention CHIMERIC PROTEINS MEDIATING TARGETED APOPTOSIS

5. Name of your agent *(if you have one)*

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# CHIMERIC PROTEINS MEDIATING TARGETED APOPTOSIS

This invention relates to chimeric cell surface proteins, nucleic acid sequences encoding such proteins, and the use of these sequences in cancer therapy and other kinds of therapy that involve the selective induction of apoptosis in particular target cell types *in vivo* or *in vitro*.

Fas (APO-1, CD95) is a member of a large family of conserved transmembrane proteins known collectively as the tumour necrosis factor receptor (TNFR) family (Baker and Reddy, 1998). Upon interaction with their respective cell surface and/or soluble ligands, for example, FasL, TNF-α, LT-α, TRAIL, RANKL/TRANCE, TWEAK/Apo-3L, a subset of these proteins including, for example, Fas, TNFR1, TRAIL-R1/DR4, TRAIL-R2/DR5, OPG, TRAMP/DR3 and DR6, induce apoptosis, a form of programmed cell death characterised by a series of biochemical events that result ultimately in the degradation of genomic DNA (Baker and Reddy, 1998). Receptor oligomerization induced by ligand binding is critical to this process (Ware et al., 1996). The cytoplasmic domain of these various pro-apoptotic proteins contains a short conserved amino acid sequence known as the "death domain" that upon receptor ligation associates with a homologous domain present within a number of adapter proteins, for example, FADD/MORT1, TRADD and RIP (Schulze-Osthoff *et al.*, 1998), triggering the activation of downstream caspases, leading ultimately to the induction of apoptosis (Nunez *et al.*, 1998).

Takebayashi *et al.*, (1996) describe a method in which chimeric proteins that incorporate the transmembrane and cytoplasmic domains of murine Fas fused in-frame to a cytoplasmic ligand-binding domain derived from the rat estrogen receptor or human retinoic acid receptor, induce the apoptotic cell death of transfected L929 and HeLa cells *in vitro* following addition of the corresponding ligand (17β-estradiol or retinoic acid). Human pancreatic carcinoma cell lines transfected with a DNA construct encoding the Fas-estrogen receptor chimera were similarly killed *in vitro* in the presence of 17β-estradiol (Kawaguchi *et al.*, 1997). In a modification to the method, the cytoplasmic ligand-binding domain in the chimeric protein was replaced with an equivalent domain derived from a mutant estrogen receptor, generating a fusion that is unable to bind estrogen, but which retains affinity for the synthetic estrogen agonist 4-hydroxytamoxifen. L929 cells transfected

with a DNA construct encoding this chimeric protein were killed *in vitro* in the presence of tamoxifen but not in the presence of 17β-estradiol (Kodaira *et al.*, 1998). Although such constructs may have utility in cancer gene therapy they lack specificity for tumour cells (as was noted in Takebayashi *et al* above). Normal tissues that express the chimeric protein will also be killed in the presence of the appropriate ligand, which in an *in vivo* setting would preferably be administered systemically. Moreover, the design of these chimeric proteins and, in particular, the cytoplasmic location of the ligand-binding domain, limits the range of potential ligands to those capable of crossing the cell membrane, for example, lipophilic hormones.

In one aspect, the invention includes a method for inducing the apoptotic death of target cells. The method includes introducing into cells a nucleic acid construct that encodes a chimeric cell surface protein, which upon interaction with an appropriate ligand initiates biochemical events that lead ultimately to cell death. The nucleic acid construct may be introduced and expressed in target cells using an expression vector that may be a plasmid, retroviral vector, adenoviral vector, adeno-associated viral vector, or the like. The method may be applied to, for example, the treatment of cancer, autoimmune disease, inflammation and other normal or abnormal conditions where the selective destruction of particular cell types is desired.

The expression and/or functional activity of various cell surface receptors are altered during the malignant process. The expression of both cell surface and soluble ligands may also be induced within the tumour microenvironment. While such changes may contribute to tumour growth, local invasion and metastasis, they may also constitute potential targets for therapeutic intervention.

The present invention provides a nucleic acid construct encoding a chimeric cell surface protein having at least three functional elements: (i) an extracellular ligand-binding domain, (ii) a membrane spanning domain, and (iii) a cytoplasmic domain that can induce cell death upon interaction of the extracellular domain with an appropriate multivalent ligand. Suitable cytoplasmic domains according to the invention include, for example, those containing one or more "death domains" derived from, or corresponding to, one or more members of the Fas/TNFR family. In a preferred embodiment of the invention, the ligand-binding domain encoded by the nucleic acid construct is derived from a receptor protein that is either differentially

activated in the target cell population, or which recognises a ligand that is differentially expressed in the vicinity of the target cell population. Crosslinking of the encoded chimeric cell surface protein by an appropriate multivalent ligand induces the apoptotic death of cells expressing the nucleic acid construct.

Nucleic acid constructs comprising the invention may be administered as a sole therapy or in combination with other therapies. For the treatment of solid tumours, nucleic acid constructs of the invention may be administered in combination with radiotherapy or photodynamic therapy, or in combination with other nucleic acid constructs or anti-tumour substances including mitotic inhibitors, for example, vinblastine, paclitaxel and docetaxel; alkylating agents, for example, cisplatin, carboplatin and cyclophosphamide; antimetabolites, for example, 5-flurourocil, cytosine arabinoside and hydroxyurea; intercalating agents, for example, adriamycin and bleomycin; enzymes, for example, aspariginase; topoisomerasé inhibitors, for example, etoposide, topotecan and irinotecan; thymidine synthase inhibitors, for example, raltitrexed; vascular-targeting agents, for example, combretastatin A4 disodium phosphate; biological response modifiers, for example, interferon; antibodies, for example, edrecolomab; and hormone agonists, for example, tamoxifen. Such combination treatment may involve simultaneous or sequential application of the individual components of the treatment.

In a preferred embodiment of the invention, the nucleic acid construct is introduced into a target cell *in vivo* or *in vitro* as part of a complete expression vector in a pharmaceutically-acceptable vehicle, either by direct administration to the target tissue (e.g. injection into the target tissue), or by systemic administration (e.g. intravenous or intraperitoneal injection). In both cases, the nucleic acid construct may be targeted to a selected tissue or cell type, for example, by incorporating it in a virion expressing a chemically or genetically altered cellular receptor that recognises a differentially expressed counter receptor, or by placing expression of the nucleic acid construct under the control of an appropriate promoter and/or enhancer element that is functional in the target cell type or tissue but not in other cell types or tissues, or under the control of a promoter and/or enhancer element that can be induced or activated locally by an appropriate stimulus (e.g. ionising radiation).

As described below, a variety of extracellular ligand-binding domains, and cytoplasmic "death domains" may be employed in the practice of the present invention.

A general example of the invention is a nucleic acid construct that encodes a chimeric cell surface protein that incorporates a cytoplasmic domain derived from Fas or another pro-apoptotic member of the Fas/TNFR family, and the extracellular ligand-binding domain of the adhesion protein CD44. CD44 is a broadly distributed cell surface glycoprotein that can function as a receptor for a variety of extracellular matrix and cell surface ligands including, for example, the glycosaminoglycans hyaluronan and chondroitin-4-sulfate (Lesley *et al.*, 1993; Cooper and Dougherty, 1995; Chiu *et al.*, 1999). In common with many other adhesion proteins, however, the ligand binding function of CD44 is not regulated simply by expression (Lesley *et al.*, 1993). Thus, while many normal cell types express CD44, only a subset of these can bind either immobilised or soluble hyaluronan (Lesley *et al.*, 1993). The hyaluronan binding function of CD44 is activated by various stimuli and is frequently induced on malignant cells (Lesley and Hyman, 1992; Lesley *et al.*, 1993; Lesley *et al.*, 1997; Sy *et al.*, 1997). While the precise mechanism involved has not yet been defined, evidence suggests that changes in the glycosylation of CD44 may be important in regulating the functional activity of the molecule (English *et al.*, 1998). For certain tumours, a correlation has been noted between CD44 expression, hyaluronan-binding function, or the expression of particular alternatively spliced CD44 isoforms, and metastatic propensity and/or poor prognosis (Cooper and Dougherty, 1995; Lesley *et al.*, 1997; Rudzki and Jothy, 1997; Sy *et al.*, 1997; Goldbrunner *et al.*, 1998; Takahashi *et al.*, 1999). Thus, nucleic acid constructs encoding chimeric proteins that incorporate a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family, and the extracellular ligand-binding domain of CD44 may be therapeutically useful, for example, in the treatment of cancer or other conditions where the destruction of cells in which CD44 is activated, is desired. It is noteworthy that the CD44 ligand hyaluronan is also differentially expressed in various tissues and that production of the molecule may be upregulated at sites of angiogenesis, inflammation, wound healing, and within certain solid tumours (Laurent and Fraser, 1992; Oksala *et al.*, 1995; Rooney *et al.*, 1995; Fraser *et al.*, 1997; Setala et al., 1999).

A second general example of the invention is a nucleic acid construct that encodes a chimeric cell surface protein that incorporates a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family, and the extracellular ligand-binding domain of the adhesion protein ICAM-1. The cell surface glycoprotein ICAM-1 functions as a ligand for the β2-integrin LFA-1 (van de Stolpe and van der Saag, 1996). Expression of ICAM-1 is induced on endothelial cells at sites of inflammation and within tumours as a result of exposure to various pro-inflammatory cytokines (Walsh and Murphy, 1992; van de Stolpe and van der Saag, 1996). Nucleic acid constructs encoding chimeric proteins that incorporate a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family and the extracellular domain of ICAM-1 may be useful in circumstances where the selective destruction of target cells in the presence of hemopoietic cells expressing LFA-1 is desirable. For example, this method could be used to effect the killing of endothelial cells within tumours or at sites of inflammation. It is noteworthy that although LFA-1 is widely expressed on hemopoietic cells, the ligand-binding function of the molecule is only induced following appropriate stimulation (Springer, 1990).

A third general example of the invention is a nucleic acid construct that encodes a chimeric cell surface protein that incorporates a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family, and the extracellular ligand-binding domain of a receptor for the cytokine vascular endothelial growth factor (VEGF), for example VEGFR1/Flt-1, VEGFR2/KDR/Flk-1 or VEGFR3/Flt-4 (Neufeld *et al.*, 1999). Various soluble ligands are known to be induced within both normal and malignant tissues in response to specific microenvironmental stimuli. Thus, the chaotic nature of the angiogenic process that occurs within solid tumours generates regions of chronic and transient hypoxia not found in normal tissues (Chaplin and Trotter, 1990; Vaupel, 1996; Brown and Giaccia, 1998). Exposure to hypoxic conditions can induce tumour cells to produce soluble mediators such as VEGF that function to induce the formation of new blood vessels (Shweiki *et al.*, 1992; Minchenko *et al.*, 1994). Nucleic acid constructs encoding chimeric proteins that incorporate a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family and an extracellular ligand-binding domain derived from a VEGF receptor, for example, VEGFR1/Flt-1, VEGFR2/KDR/Flk-1 or VEGFR3/Flt-4, may be therapeutically useful in

circumstances where the destruction of normal or malignant target cells in the presence of VEGF is desirable.

A fourth general example of the invention is a nucleic acid construct that encodes a chimeric cell surface protein that incorporates a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family, and the extracellular ligand-binding domain of a receptor for the cytokine platelet-derived growth factor (PDGF). Restenosis is a significant clinical problem associated with the trauma induced by mechanical procedures such as coronary angioplasty and stenting that are commonly used in the treatment of vascular occlusive disease. Vascular smooth muscle cell proliferation plays a critical role in the development of these conditions and in the evolution of spontaneous atherosclerosis, hypertension-related arteriosclerosis, and venous bypass graft arteriosclerosis (Zou *et al.*, 1998). PDGF, is a potent chemotactic and mitogenic agent for vascular smooth muscle cells and recent studies have implicated this molecule in the development of these various vascular lesions (Abe *et al.*, 1998). Thus, nucleic acid constructs encoding chimeric proteins that incorporate a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family and the extracellular domain of the alpha or beta PDGF receptors, may be therapeutically useful, for example, in the treatment of atherosclerosis or restenosis, or other conditions where the destruction of target cells in the presence of PDGF is desirable.

A fifth general example of the invention is a nucleic acid construct that encodes a chimeric cell surface protein that incorporates a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family, and the extracellular ligand-binding domain of a receptor for the cytokine epidermal growth factor (EGF). Members of EGF superfamily including, for example, EGF and Cripto-1, play an important role in regulating the proliferation and differentiation of both normal and malignant epithelial cells (Jones *et al.*, 1999; Salomon *et al.*, 1999; Thomas *et al.*, 1999). High levels of EGF and related proteins can be detected within various solid malignancies including, for example, those of the breast, ovary and stomach. EGF induces homodimerization of the EGF receptor (EGFR) and heterodimerization of the EGFR and ErbB2 (Wang *et al.*, 1999). Thus, nucleic acid constructs encoding chimeric proteins that incorporate a cytoplasmic domain derived from Fas or another member of the Fas/TNFR family and the extracellular ligand-binding domain of an EGF receptor, may be therapeutically useful, for example, in the

treatment of epithelial malignancies, or other conditions where the destruction of target cells in the presence of EGF is desirable.

Nucleic acid sequences encoding the ligand-binding domains and counter-receptors discussed above represent exemplary domains useful in the practice of the present invention. It will be appreciated, however, that following the teachings and guidance of the present specification, one of skill in the art may select other sequences suitable for use with the present invention, and that the use of such sequences is considered to be within the scope of the present invention.

Those skilled in the art will also recognise that cytoplasmic domains derived from the various members of the Fas/TNFR family may have different activities in different target cell types. It will also be appreciated that cytoplasmic domains lacking classical "death domains" of the type seen in the various members of the Fas/TNFR family and which kill cells by a different mechanism may nevertheless prove suitable for use with the present invention

The following examples illustrate but are in no way are intended to limit the present invention, and that the use of such sequences is considered to be within the scope of the present invention.

## EXAMPLES

Example 1

### Cytotoxic activity of CD44-Fas chimeric proteins

Vector Construction

A full length CD44H cDNA was isolated from pCDM8.CD44H clone 2.3 (Dougherty *et al*, 1991) by digestion with HindIII and NotI and the fragment obtained cloned into the HindIII-NotI sites of the episomal expression vector pCEP4 (Invitrogen) generating a plasmid designated pCEP4.CD44H. Digestion of pCEP4.CD44H with XhoI released a fragment containing the full length CD44H cDNA, which was blunted using T4 DNA polymerase and cloned into the EcoRV site of pZErO2 (Invitrogen). Orientation of the insert was determined by digestion with a panel of

restriction enzymes and an appropriate clone digested with EcoRI and Not I to release the full length CD44H cDNA in which the 3' end of the gene is located adjacent to the EcoRI site. This fragment was cloned into the EcoRI-NotI sites of pBluescript (KS+) (Stratagene) generating a vector designated pBS.CD44H.

mRNA was isolated from approximately 4 x $10^7$ Jurkat cells using the Stratagene mRNA Isolation Kit (Stratagene). The mRNA was reverse transcribed and cDNA synthesized using the Pharmacia cDNA synthesis kit (Pharmacia) as per the manufacturers instructions using a random hexanucleotide primer. A 'full-length' human Fas cDNA ($Fas^{FL}$) or cDNA fragments encoding the transmembrane and cytoplasmic domains of human Fas ($Fas^{TM/CYTO}$) or only the cytoplasmic domain of the molecule ($Fas^{CYTO}$) were generated by polymerase chain reaction (PCR) using the following primer pairs designed on the basis of published Fas sequences (Itoh *et al.*, 1991).

$Fas^{FL}$

5' primer 5' GCGGAATTCAGGGGCGGGCACTGGCAC 3'
EcoR1

3' primer 5' GGCTCGAGAATCTTTTCAAACACTAATTGC 3'
Xho1

$Fas^{TM/CYTO}$

5' primer 5' AACGTGATCATCCTTTGTCTTCTTCTTTTG 3'
BclI

3' primer 5' GGCTCGAGAATCTTTTCAAACACTAATTGC 3'
Xho1

$Fas^{CYTO}$

5' primer 5' GCCCGGGGTGAAGAGAAAGGAAGTACAG 3'
SmaI

3' primer 5' GGCTCGAGAATCTTTTCAAACACTAATTGC 3'
Xho1

The underlined base pairs in the 5' $Fas^{TM/CYTO}$ primer are not found in Fas and were introduced to maintain the correct reading frame. The restriction enzyme sites used in subsequent cloning steps are indicated in bold.

PCR reactions (94°C for 30s, 50°C for 30s and 72°C for 1 min; 40 cycles) were carried out in an OmniGene Thermacycler (Hybaid) using Ampli-Taq (Perkin-Elmer). PCR products were blunted using T4 DNA polymerase and cloned into the EcoRV site of pZErO2 generating vectors designated $pZErO2.Fas^{FL}$, $pZErO.Fas^{TM/CYTO}$ and $pZErO.Fas^{CYTO}$.

$pZErO2.Fas^{FL}$ was digested with EcoRI-XhoI to release the full length Fas cDNA which was then ligated into the EcoRI-XhoI sites of pBluescript (KS+) generating the vector $pBS.Fas^{FL}$. Digestion of $pBS.Fas^{FL}$ with NotI and XhoI released a fragment containing the full length Fas cDNA which was then ligated into the corresponding NotI-XhoI sites of the episomal expression vector pCEP4 (Invitrogen). The major features of this vector are illustrated in Figure 1.

To generate a nucleic acid construct encoding a chimeric protein containing the extracellular domain of CD44H and the transmembrane and cytoplasmic domains of human Fas ($CD44^{EXTRA}$-$Fas^{TM/CYTO}$), pBS.CD44H was digested with BclI and XhoI to remove the transmembrane and cytoplasmic domain of CD44H and a BclI-XhoI fragment derived from $pZErO.Fas^{TM/CYTO}$ containing the transmembrane and cytoplasmic domains of Fas was ligated into the corresponding sites in the plasmid generating a vector designated $pBS.CD44^{EXTRA}$-$Fas^{TM/CYTO}$. The complete nucleic acid sequence and predicted amino acid sequence of $CD44^{EXTRA}$-$Fas^{TM/CYTO}$ are shown in Figure 2.

To generate a nucleic acid construct encoding a chimeric protein containing the extracellular and transmembrane domains of CD44H and the cytoplasmic domain of human Fas ($CD44^{EXTRA/TM}$-$Fas^{CYTO}$) pBS.CD44H was digested with NotI and EcoRI to release the full length CD44H cDNA which was then partially digested with HincII to obtain a NotI-HincII fragment that contained only the extracellular and transmembrane domains of the CD44H molecule. $pZErO.Fas^{CYTO}$ was digested with NotI and SmaI and the NotI-HincII fragment containing the extracellular and transmembrane domains of CD44H was ligated into the plasmid generating a vector designated $pZErO.CD44^{EXTRA/TM}$-$Fas^{CYTO}$. The complete nucleic acid sequence and

predicted amino acid sequence of $CD44^{EXTRA/TM}$-$Fas^{CYTO}$ are shown in Figure 3.

In order to test the functional activity of the nucleic acid constructs, full length $CD44^{EXTRA}$-$Fas^{TM/CYTO}$ and $CD44^{EXTRA/TM}$-$Fas^{CYTO}$ chimeric cDNAs were isolated by digestion of pBS.$CD44^{EXTRA}$-$Fas^{TM/CYTO}$ and pZErO.$CD44^{EXTRA/TM}$-$Fas^{CYTO}$ with NotI and XhoI. The fragments obtained were then cloned into the NotI-XhoI sites of the episomal expression vector pCEP4 (Invitrogen) generating vectors designated pCEP4.$CD44^{EXTRA}$-$Fas^{TM/CYTO}$ and pCEP4.$CD44^{EXTRA/TM}$-$Fas^{CYTO}$. The major features of both plasmids are illustrated in Figure 1.

Cell lines and culture conditions

The cell lines K562 (human erythroleukemia), PC-3 (human prostatic adenocarcinoma) and ECV304 (a variant of the T28 bladder carcinoma) were obtained from the American Type Culture Collection (ATCC). All three were maintained at 37°C in an atmosphere containing 5% $CO_2$ in Dulbecco's Minimal Essential Medium (DMEM) supplemented with fetal bovine serum (10%), glutamine (2mM), penicillin (50 units/ml), and streptomycin sulfate (50 µg/ml).

Clonagenic assays

Tumour cell lines were transfected with plasmid DNA by electroporation using the BTX ECM 600 Electroporator System (BTX). Briefly, log-phase cultures were harvested and cells resuspended in phosphate buffered saline (PBS) at a final concentration of 1 x $10^7$ cells/ml. 15 µg of plasmid DNA (pCEP4, pCEP4.$Fas^{FL}$, pCEP4.CD44H, pCEP4.$CD44^{EXTRA}$-$Fas^{TM/CYTO}$ or pCEP4.$CD44^{EXTRA/TM}$-$Fas^{CYTO}$) were added to a 400 µl aliquot of each cell suspension, transferred to a 2 mm gap cuvette and electroporated using the following conditions. ECV304:- resistance setting R3, 280 V, 500 µF. The time constants obtained ranged from 3.0-3.5 ms. PC-3:- resistance setting R3, 280 V, 300 µF. The time constants obtained ranged from 2.0-2.5 ms. Immediately after electroporation, cells were resuspended in 30 ml tissue culture medium, plated in a 15 cm Integrid dish (Falcon) and incubated at 37°C in an atmosphere containing 5% $CO_2$.

Hygromycin B (Sigma) was added to each culture 48 h later at a final concentration of 250 μg/ml. Plates were incubated undisturbed for 18-21 days after which time the tissue culture supernatant was removed and the number of colonies derived from single cells that survived the treatment, were determined after staining in a solution containing 1% (w/v) methylene blue in methanol. Adherent cell lines such as ECV304 and PC-3 constitutively produce hyaluronan, which is found associated with the cell surface bound to proteins such as CD44 forming a pericellular coat (Laurent and Fraser, 1992; Knudson *et al.*, 1996; Fraser *et al.*, 1997). As shown in Figure 4, for both ECV304 and PC-3, overexpression of CD44H produced only a modest decrease in the number of hygromycin resistant colonies relative to cells transfected with the pCEP4 vector alone. In contrast, dramatic reductions in colony formation were observed following transfection of these cell lines with plasmids encoding either $CD44^{EXTRA}$-$Fas^{TM/CYTO}$ or $CD44^{EXTRA/TM}$-$Fas^{CYTO}$. Although substantial numbers of colonies were generated following transfection of ECV304 cells with pCEP4.$Fas^{FL}$, very few colonies resulted when PC-3 cells with transfected with the same vector. This finding is in agreement with previous studies that demonstrated constitutive production of FasL by PC-3 (Liu *et al.*, 1998).

Hemopoietic cells such as K562 generally produce very low or undetectable levels of hyaluronan (Laurent and Fraser, 1992; Fraser *et al.*, 1997).

Establishment of stably transfected K562 cells

K562 cells do not express CD44 and can be used to characterize the expression and functional activity of chimeric CD44-Fas proteins in the absence of a contribution from the endogenous CD44 protein. K562 cells were transfected with plasmid DNA by electroporation using the BTX ECM 600 Electroporator System (BTX, San Diego, CA) as described above. Briefly, log-phase cultures were harvested and cells resuspended in phosphate buffered saline (PBS) at a final concentration of $1 \times 10^7$ cells/ml. 15 μg of plasmid DNA (pCEP4, pCEP4.$Fas^{FL}$, pCEP4.CD44H, pCEP4.$CD44^{EXTRA}$-$Fas^{TM/CYTO}$ or pCEP4.$CD44^{EXTRA/TM}$-$Fas^{CYTO}$) were added to a 400 μl aliquot of each cell suspension, transferred to a 2 mm gap cuvette and electroporated using resistance setting R3, 280 V, 500 μF. The time constants obtained

ranged from 3.0-3.5 ms. Immediately after electroporation, the transfected cells were resuspended in 30 ml tissue culture medium, plated in a 15 cm Integrid dish and incubated at 37°C in an atmosphere containing 5% $CO_2$. Hygromycin B (Sigma) was added 48 hours after electroporation at a final concentration of 250 μg/ml and transfected cells selected for a minimum of 14 days before being further analysed.

Fluorescent antibody cell sorter (FACS) analysis of stably transfected K562

Expression of Fas, CD44H and chimeric $CD44^{EXTRA}$-$Fas^{TM/CYTO}$ and $CD44^{EXTRA/TM}$-$Fas^{CYTO}$ proteins on the surface of transfected K562 cells was determined by FACS analysis. Briefly, $5x10^5$ cells were incubated with anti-CD44 mAb 4A4 tissue culture supernatant (Droll *et al.*, 1995), or the mouse anti-human Fas mAb DX2 (PharMingen) at a final concentration of 5 μg/ml or media alone, for 30 min at 4°C. After 3 washes with ice-cold Hank's balanced salt solution (HBSS) containing 2% FCS (HBSS+2% FCS), the cells were stained for a further 30 min at 4°C with an FITC-conjugated goat anti-mouse antibody (PharMingen) at a final concentration of 5 μg/ml in HBSS+2% FCS. Following extensive washing, cells were resuspended in HBSS+2% FCS containing 1 μg/ml propidium iodide (PI) (Sigma) to facilitate the identification and exclusion of dead cells, and analyzed on a FACScan (Becton Dickinson). As shown in Figure 5, Fas, CD44H and the chimeric proteins $CD44^{EXTRA}$-$Fas^{TM/CYTO}$ and $CD44^{EXTRA/TM}$-$Fas^{CYTO}$ could all be detected on the surface of the corresponding transfected K562 cells.

Induction of apoptosis by binding to immobilized hyaluronan

In order to determine whether cells expressing chimeric CD44-Fas proteins undergo apoptosis upon ligand binding, the wells of 6 well tissue culture plates (Falcon) were coated overnight at 4°C with human placental hyaluronan (Sigma) (5mg/ml in PBS). Unbound hyaluronan was decanted and the wells washed 5 times with PBS and twice with medium. $5x10^6$ transfected K562 cells in a final volume of 3 ml HBSS were added to each dish. After incubation for 10 min at 37°C, non-adherent cells were removed by gently washing with medium. K562 cells transfected with CD44H or the chimeric

proteins $CD44^{EXTRA}$-$Fas^{TM/CYTO}$ or $CD44^{EXTRA/TM}$-$Fas^{CYTO}$ bound avidly to the hyaluronan-coated dishes. Equivalent cells transfected with the pCEP4 vector alone or with pCEP4.$Fas^{FL}$ did not adhere reflecting the absence of CD44 or other hyaluronan binding proteins.

The induction of apoptosis upon adhesion to hyaluronan was determined using the method of Fraker *et al.*, (1995). Briefly, transfected K562 cells that had been allowed to adhere to plastic surfaces coated with hyaluronan for 1 h were recovered by gentle pipetting. Approximately 2 x $10^6$ cells were then aliquoted into sample tubes (Falcon 2099, Becton Dickinson), pelleted by centrifugation at 350g for 10 min, washed once in HBSS, and then resuspended in 2 ml ice cold 70% ethanol with rapid but gentle mixing. Cells were fixed by incubation at –20°C for at least 4 h, centrifuged at 400 g for 10 min, washed once in HBSS and resuspended in 1 ml DNA staining solution (PBS, pH 7.4, containing 0.1% Triton X-100, 0.1 mM EDTA pH7.4, 0.05 mg/ml RNase A, and 50 µg/ml propidium iodide). Cells were stained for at least 4 h in the dark at room temperature and the apoptotic fraction determined by FACS analysis (FACSCalibur, Becton Dickinson). Briefly, data were collected for at least 10,000 events and FL2 histograms generated. Using the CellQuest software package (Becton Dickinson) gates were set to calculate the percentage of hypodiploid cells (i.e. those cells with a sub $G_0/G_1$ DNA content). As shown in Table I, a substantial proportion of transfected K562 cells expressing either the of chimeric proteins $CD44^{EXTRA}$-$Fas^{TM/CYTO}$ or $CD44^{EXTRA/TM}$-$Fas^{CYTO}$ underwent apoptosis following a brief 1 h adhesion to hyaluronan. In contrast, K562 cells expressing CD44H adhered to hyaluronan but remained largely viable.

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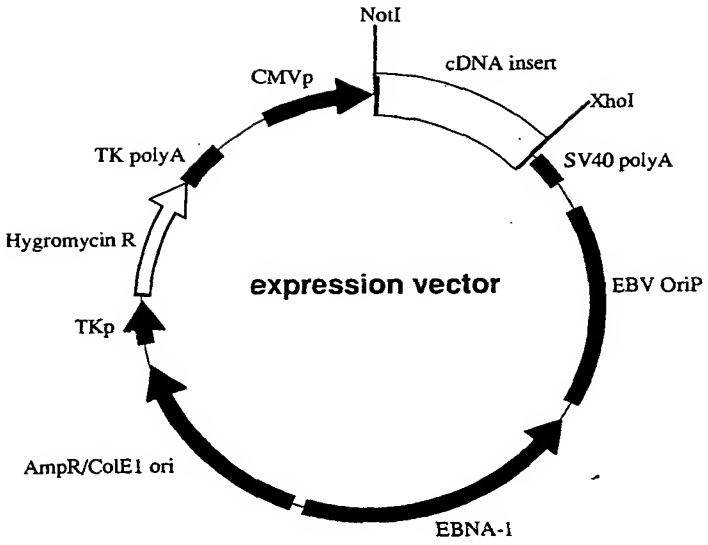
**Table I**

| | % apoptotic cells | |
|---|---|---|
| | Starting Culture | 1 hr binding |
| K562:CD44H | 7.6% | 3.0% |
| K562:CD44H$^{EXTRA}$FAS$^{TM/CYTO}$ | 2.4% | 58.5% |
| K562:CD44H$^{EXTRA/TM}$FAS$^{CYTO}$ | 3.5% | 40.7% |

# Figure 1

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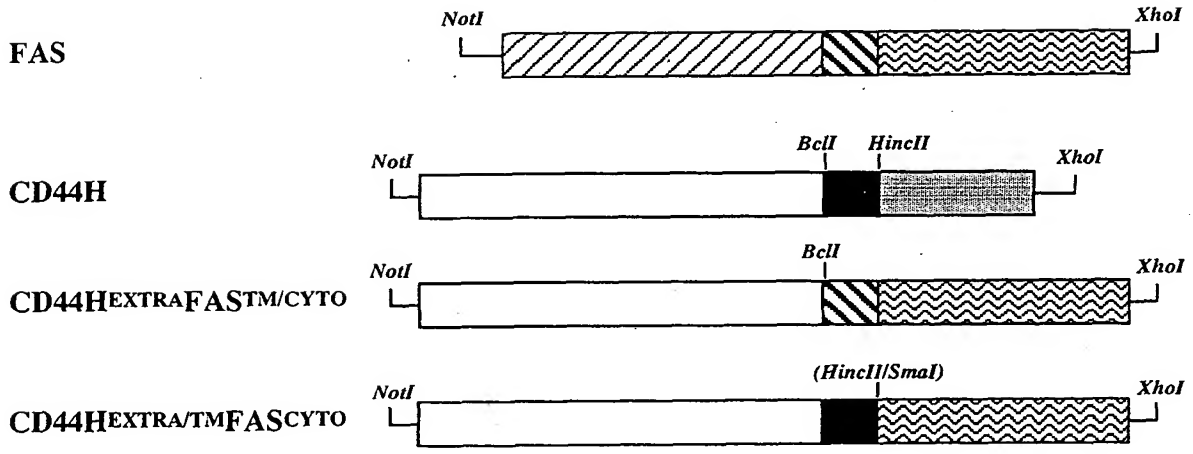

# Figure 2



## $CD44H^{EXTRA}$-$FAS^{TM/CYTO}$

```
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45
GCCGGCCCCTGCCCCGCGCCCAGGGATCCTCCAGCTCCTTTCGCCCGCGCCCTCCGTTCGCTCCGGACACC ATG GAC
                                                                                                        Met Asp
                                                                                                        -19
122
AAG TTT TGG TGG CAC GCA GCC TGG GGA CTC TGC CTC GTG CCG CTG AGC CTG GCG CAG ATC
Lys Phe Trp Trp His Ala Ala Trp Gly Leu Cys Leu Val Pro Leu Ser Leu Ala Gln Ile
                                                                                                        +1
182
GAT TTG AAT ATA ACC TGC CGC TTT GCA GGT GTA TTC CAC GTG GAG AAA AAT GGT CGC TAC
Asp Leu Asn Ile Thr Cys Arg Phe Ala Gly Val Phe His Val Glu Lys Asn Gly Arg Tyr
                                                                                                        23
242
AGC ATC TCT CGG ACG GAG GCC GCT GAC CTC TGC AAG GCT TTC AAT AGC ACC TTG CCC ACA
Ser Ile Ser Arg Thr Glu Ala Ala Asp Leu Cys Lys Ala Phe Asn Ser Thr Leu Pro Thr
                                                                                                        43
302
ATG GCC CAG ATG GAG AAA GCT CTG AGC ATC GGA TTT GAG ACC TGC AGG TAT GGG TTC ATA
Met Ala Gln Met Glu Lys Ala Leu Ser Ile Gly Phe Glu Thr Cys Arg Tyr Gly Phe Ile
                                                                                                        63
362
GAA GGG CAT GTG GTG ATT CCC CGG ATC CAC CCC AAC TCC ATC TGT GCA GCA AAC AAC ACA
Glu Gly His Val Val Ile Pro Arg Ile His Pro Asn Ser Ile Cys Ala Ala Asn Asn Thr
                                                                                                        83
422
GGG GTG TAC ATC CTC ACA TAC AAC ACC TCC CAG TAT GAC ACA TAT TGC TTC AAT GCT TCA
Gly Val Tyr Ile Leu Thr Tyr Asn Thr Ser Gln Tyr Asp Thr Tyr Cys Phe Asn Ala Ser
                                                                                                        103
482
GCT CCA CCT GAA GAA GAT TGT ACA TCA GTC ACA GAC CTG CCC AAT GCC TTT GAT GGA CCA
Ala Pro Pro Glu Glu Asp Cys Thr Ser Val Thr Asp Leu Pro Asn Ala Phe Asp Gly Pro
                                                                                                        123
542
ATT ACC ATA ACT ATT GTT AAC CGT GAT GGC ACC CGC TAT GTC CAG AAA GGA GAA TAC AGA
Ile Thr Ile Thr Ile Val Asn Arg Asp Gly Thr Arg Tyr Val Gln Lys Gly Glu Tyr Arg
                                                                                                        143
602
ACG AAT CCT GAA GAC ATC TAC CCC AGC AAC CCT ACT GAT GAT GAC GTG AGC AGC GGC TCC
Thr Asn Pro Glu Asp Ile Tyr Pro Ser Asn Pro Thr Asp Asp Asp Val Ser Ser Gly Ser
                                                                                                        163
662
TCC AGT GAA AGG AGC AGC ACT TCA GGA GGT TAC ATC TTT TAC ACC TTT TCT ACT GTA CAC
Ser Ser Glu Arg Ser Ser Thr Ser Gly Gly Tyr Ile Phe Tyr Thr Phe Ser Thr Val His
                                                                                                        183
722
CCC ATC CCA GAC GAA GAC AGT CCC TGG ATC ACC GAC AGC ACA GAC AGA ATC CCT GCT ACC
Pro Ile Pro Asp Glu Asp Ser Pro Trp Ile Thr Asp Ser Thr Asp Arg Ile Pro Ala Thr
                                                                                                        203
782
AGA GAC CAA GAC ACA TTC CAC CCC AGT GGG GGG TCC CAT ACC ACT CAT GGA TCT GAA TCA
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                                                                                                        223
```

# Figure 2



842
GAT GGA CAC TCA CAT GGG AGT CAA GAA GGT GGA GCA AAC ACA ACC TCT GGT CCT ATA AGG
Asp Gly His Ser His Gly Ser Gln Glu Gly Gly Ala Asn Thr Thr Ser Gly Pro Ile Arg
243

902 *BclI*
ACA CCC CAA ATT CCA GAA TGG CTG ATC ATC CTT TGT CTT CTT CTT TTG CCA ATT CCA CTA
Thr Pro Gln Ile Pro Glu Trp Leu Ile Ile Leu Cys Leu Leu Leu Leu Pro Ile Pro Leu
263

962
ATT GTT TGG GTG AAG AGA AAG GAA GTA CAG AAA ACA TGC AGA AAG CAC AGA AAG GAA AAC
Ile Val Trp Val Lys Arg Lys Glu Val Gln Lys Thr Cys Arg Lys His Arg Lys Glu Asn
283

1022
CAA GGT TCT CAT GAA TCT CCA ACC TTA AAT CCT GAA ACA GTG GCA ATA AAT TTA TCT GAT
Gln Gly Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr Val Ala Ile Asn Leu Ser Asp
303

1082
GTT GAC TTG AGT AAA TAT ATC ACC ACT ATT GCT GGA GTC ATG ACA CTA AGT CAA GTT AAA
Val Asp Leu Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met Thr Leu Ser Gln Val Lys
323

1142
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343

1202
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Val Gln Asp Thr Ala Glu Gln Lys Val Gln Leu Leu Arg Asn Trp His Gln Leu His Gly
363

1262
AAG AAA GAA GCG TAT GAC ACA TTG ATT AAA GAT CTC AAA AAA GCC AAT CTT TGT ACT CTT
Lys Lys Glu Ala Tyr Asp Thr Leu Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu
383

1322 #
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Ala Gly Lys Ile Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser Asp Ser Glu Asn Ser Asn
403

1382
TTC AGA AAT GAA ATC CAA AGC TTG GTC TAG AGTGAAAAACAACAAATTCAGTTCTGAGTATATGCAATT
Phe Arg Asn Glu Ile Gln Ser Leu Val ***

1451
AGTGTTTGAAAAGATTCT

# Figure 3



## $CD44H^{EXTRA/TM}$-$FAS^{CYTO}$

```
                                                                1
                                                                CCAGCCTCTGCCAGGTTCGGTCCGCCATCCTCGTCCCGTCCTCC
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GCCGGCCCCTGCCCCGCGCCCAGGGATCCTCCAGCTCCTTTCGCCCGCGCCCTCCGTTCGCTCCGGACACC ATG GAC
                                                                                Met Asp
                                                                                -19
122
AAG TTT TGG TGG CAC GCA GCC TGG GGA CTC TGC CTC GTG CCG CTG AGC CTG GCG CAG ATC
Lys Phe Trp Trp His Ala Ala Trp Gly Leu Cys Leu Val Pro Leu Ser Leu Ala Gln Ile
                                                                          +1
182
GAT TTG AAT ATA ACC TGC CGC TTT GCA GGT GTA TTC CAC GTG GAG AAA AAT GGT CGC TAC
Asp Leu Asn Ile Thr Cys Arg Phe Ala Gly Val Phe His Val Glu Lys Asn Gly Arg Tyr
                                                                                23
242
AGC ATC TCT CGG ACG GAG GCC GCT GAC CTC TGC AAG GCT TTC AAT AGC ACC TTG CCC ACA
Ser Ile Ser Arg Thr Glu Ala Ala Asp Leu Cys Lys Ala Phe Asn Ser Thr Leu Pro Thr
                                                                                43
302
ATG GCC CAG ATG GAG AAA GCT CTG AGC ATC GGA TTT GAG ACC TGC AGG TAT GGG TTC ATA
Met Ala Gln Met Glu Lys Ala Leu Ser Ile Gly Phe Glu Thr Cys Arg Tyr Gly Phe Ile
                                                                                63
362
GAA GGG CAT GTG GTG ATT CCC CGG ATC CAC CCC AAC TCC ATC TGT GCA GCA AAC AAC ACA
Glu Gly His Val Val Ile Pro Arg Ile His Pro Asn Ser Ile Cys Ala Ala Asn Asn Thr
                                                                                83
422
GGG GTG TAC ATC CTC ACA TAC AAC ACC TCC CAG TAT GAC ACA TAT TGC TTC AAT GCT TCA
Gly Val Tyr Ile Leu Thr Tyr Asn Thr Ser Gln Tyr Asp Thr Tyr Cys Phe Asn Ala Ser
                                                                                103
482
GCT CCA CCT GAA GAA GAT TGT ACA TCA GTC ACA GAC CTG CCC AAT GCC TTT GAT GGA CCA
Ala Pro Pro Glu Glu Asp Cys Thr Ser Val Thr Asp Leu Pro Asn Ala Phe Asp Gly Pro
                                                                                123
542
ATT ACC ATA ACT ATT GTT AAC CGT GAT GGC ACC CGC TAT GTC CAG AAA GGA GAA TAC AGA
Ile Thr Ile Thr Ile Val Asn Arg Asp Gly Thr Arg Tyr Val Gln Lys Gly Glu Tyr Arg
                                                                                143
602
ACG AAT CCT GAA GAC ATC TAC CCC AGC AAC CCT ACT GAT GAT GAC GTG AGC AGC GGC TCC
Thr Asn Pro Glu Asp Ile Tyr Pro Ser Asn Pro Thr Asp Asp Asp Val Ser Ser Gly Ser
                                                                                163
662
TCC AGT GAA AGG AGC AGC ACT TCA GGA GGT TAC ATC TTT TAC ACC TTT TCT ACT GTA CAC
Ser Ser Glu Arg Ser Ser Thr Ser Gly Gly Tyr Ile Phe Tyr Thr Phe Ser Thr Val His
                                                                                183
722
CCC ATC CCA GAC GAA GAC AGT CCC TGG ATC ACC GAC AGC ACA GAC AGA ATC CCT GCT ACC
Pro Ile Pro Asp Glu Asp Ser Pro Trp Ile Thr Asp Ser Thr Asp Arg Ile Pro Ala Thr
                                                                                203
782
AGA GAC CAA GAC ACA TTC CAC CCC AGT GGG GGG TCC CAT ACC ACT CAT GGA TCT GAA TCA
Arg Asp Gln Asp Thr Phe His Pro Ser Gly Gly Ser His Thr Thr His Gly Ser Glu Ser
                                                                                223
```

# Figure 3



842
GAT GGA CAC TCA CAT GGG AGT CAA GAA GGT GGA GCA AAC ACA ACC TCT GGT CCT ATA AGG
Asp Gly His Ser His Gly Ser Gln Glu Gly Gly Ala Asn Thr Thr Ser Gly Pro Ile Arg
243

902
ACA CCC CAA ATT CCA GAA TGG CTG ATC ATC TTG GCA TCC CTC TTG GCC TTG GCT TTG ATT
Thr Pro Gln Ile Pro Glu **Trp Leu Ile Ile Leu Ala Ser Leu Leu Ala Leu Ala Leu Ile**
263

962 junction
CTT GCA GTT TGC ATT GCA GTC *GGG GTG AAG AGA AAG GAA GTA CAG AAA ACA TGC AGA AAG*
**Leu Ala Val Cys Ile Ala Val Gly Val** *Lys Arg Lys Glu Val Gln Lys Thr Cys Arg Lys*
283

1022
*CAC AGA AAG GAA AAC CAA GGT TCT CAT GAA TCT CCA ACC TTA AAT CCT GAA ACA GTG GCA*
*His Arg Lys Glu Asn Gln Gly Ser His Glu Ser Pro Thr Leu Asn Pro Glu Thr Val Ala*
303

1082
*ATA AAT TTA TCT GAT GTT GAC TTG AGT AAA TAT ATC ACC ACT ATT GCT GGA GTC ATG ACA*
*Ile Asn Leu Ser Asp Val Asp Leu Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met Thr*
323

1142
*CTA AGT CAA GTT AAA GGC TTT GTT CGA AAG AAT GGT GTC AAT GAA GCC AAA ATA GAT GAG*
*Leu Ser Gln Val Lys Gly Phe Val Arg Lys Asn Gly Val Asn Glu Ala Lys Ile Asp Glu*
343

1202
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*Ile Lys Asn Asp Asn Val Gln Asp Thr Ala Glu Gln Lys Val Gln Leu Leu Arg Asn Trp*
363

1262
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*His Gln Leu His Gly Lys Lys Glu Ala Tyr Asp Thr Leu Ile Lys Asp Leu Lys Lys Ala*
383

1322 #
*AAT CTT TGT ACT CTT GCA GGG AAA ATT CAG ACT ATC ATC CTC AAG GAC ATT ACT AGT GAC*
*Asn Leu Cys Thr Leu Ala Gly Lys Ile Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser Asp*
403

1382
*TCA GAA AAT TCA AAC TTC AGA AAT GAA ATC CAA AGC TTG GTC TAG AGTGAAAAACAACAAATTC*
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1442
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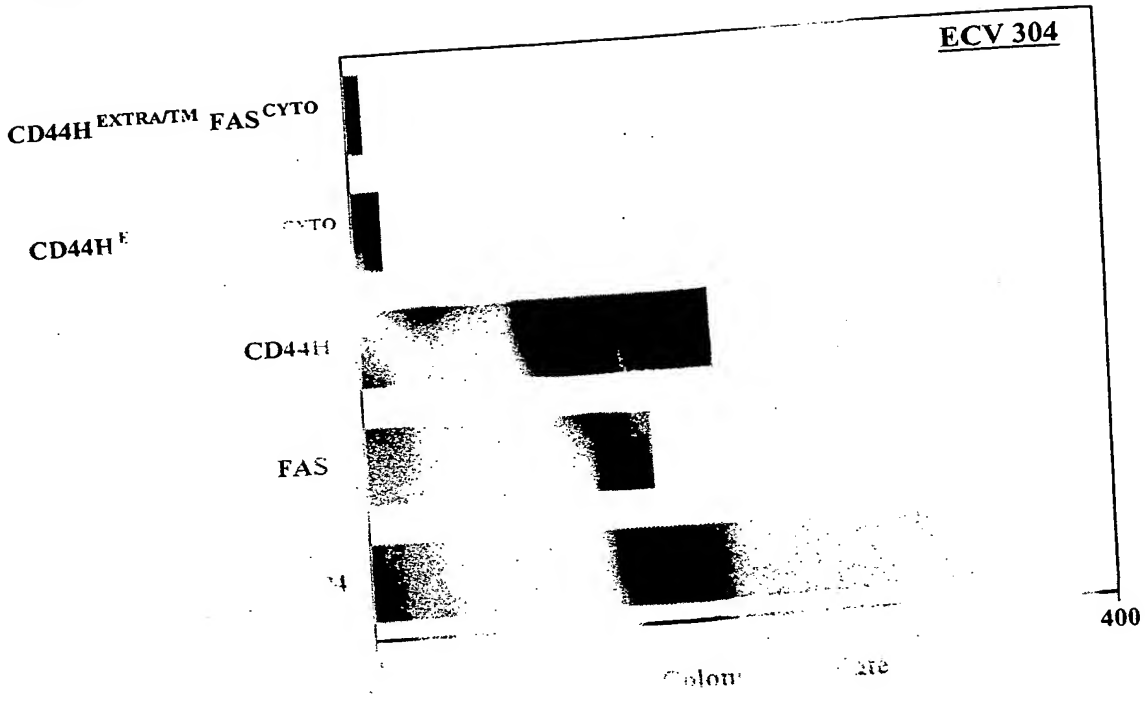
# Figure 4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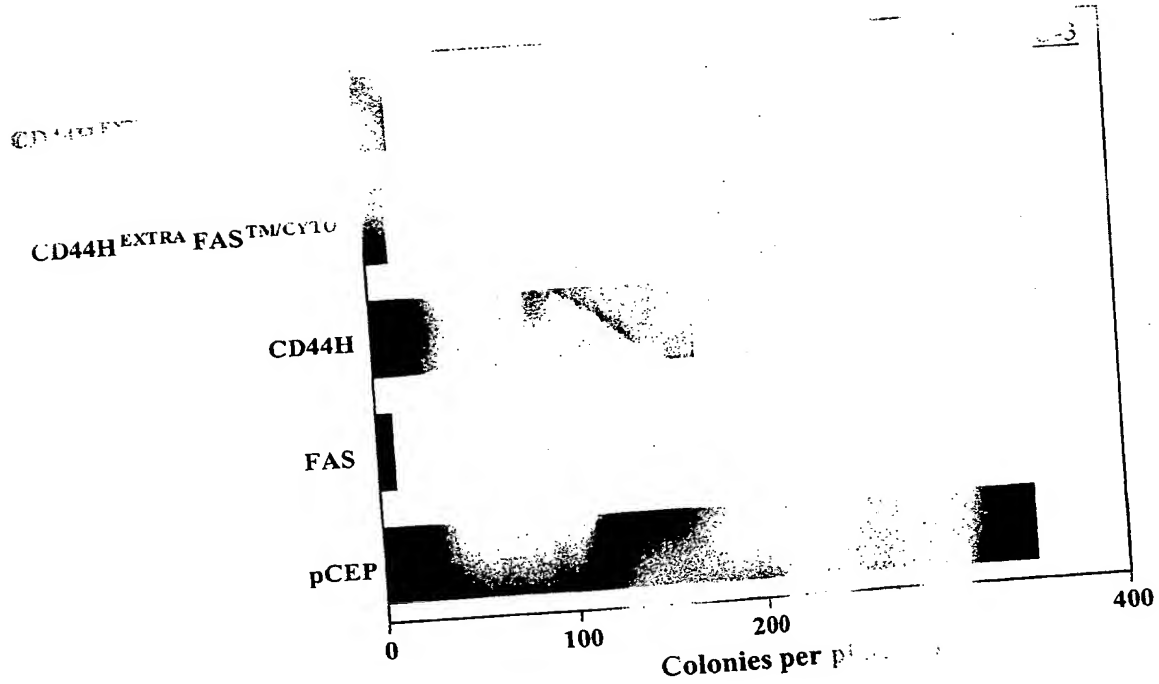
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CD44H
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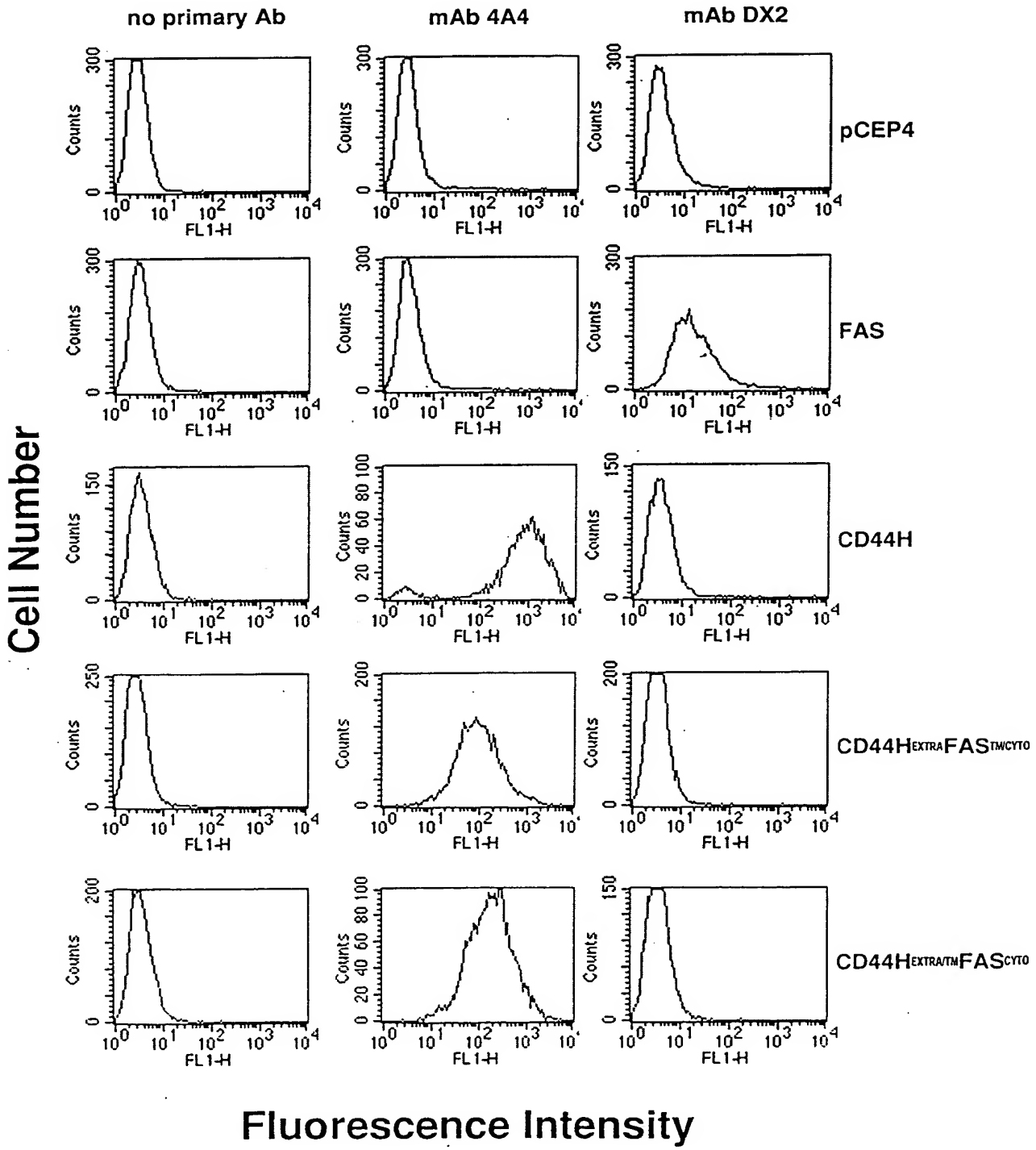
pCEP
0
100
200
400

Figure 5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CD44HEXTRA/TMFASCYTO
Counts
FL1-H
Cell Number
Fluorescence Intensity

Mewburn Ellis.

PCT GB 00 02449

26/6/2000.